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COOPERATIVE AGREEMENT NO: DAMD17-93-V-3008

TITLE: AUTORADIOGRAPHIC DISTRIBUTION AND APPLIED PHARMACOLOGICAL CHARACTERISTICS OF DEXTROMETHORPHAN AND RELATED ANTITISSUE/ANTICONVULSANT DRUGS AND NOVEL ANALOGS

SUBTITLE: Autoradiographical Distribution of ^{3}H-Dextromethorphan Binding Sites in Rat and Human Brain

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REPORT DATE: October 1, 1994

TYPE OF REPORT: Midterm Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1, 1994	Midterm Report (9/27/93-9/26/94)

4. TITLE AND SUBTITLE Autoradiographic Distribution and Applied Pharmacological Characteristics of Dextromethorphan and related Antitissue/Anticonvulsant Drugs and Novel Analogs

5. FUNDING NUMBERS
Cooperative Agreement
DAMD17-93-V-3008

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Subtitle: Autoradiographical Distribution of ^{3}H-Dextromethorphan Binding Sites in Rat and Human Brain

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Dextromethorphan is an antitussive compound with anticonvulsant and neuroprotective properties arising from actions within the central nervous system of mammals. In the present study we have determined the distribution of [^{3}H]-dextromethorphan ([^{3}H]-DM) binding sites in normal rat brain and 72 hours after middle cerebral artery occlusion. The pattern of distribution did not correlate with that of the excitatory amino acid, N-methyl-D-aspartate (NMDA), with which an association has previously been suggested on the basis of a weak pharmacological interaction. A decrease in binding within certain regions of the cerebral cortex of occluded animals was observed. However, this was limited and may be a consequence of the possible intracellular location of the DM sites or simply due to the period allowed between the occlusion and preparing the brain sections. At 72 hours [^{3}H]-DM binding was clearly not a marker for the degree of neuronal damage. At autoradiographic technique is also being developed for examining the binding of [^{3}H]-DM in post mortem human whole brain sections. Whole coronal sections have been prepared and binding of [^{3}H]-DM obtained. However, further developmental studies are required to obtain the optimal conditions for obtaining a distribution pattern of [^{3}H]-DM sites.

14. SUBJECT TERMS
Drugs, Anticonvulsants, Neuroprotective, Dextromethorphan, Autoradiography, RAD V, RAD II

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Distribution /		
Availability Codes		
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PI - Signature   24 October 1994
Date

AUTORADIOGRAPHICAL DISTRIBUTION OF ^{3}H-DEXTROMETHORPHAN BINDING SITES IN RAT AND HUMAN BRAIN

Introduction

Dextromethorphan (DM) is a non-opioid cough suppressant which is the dextrorotatory isomer of the opioid levorphanol. However, it exhibits little or none of the actions of the opioids with no analgesic activity or respiratory depressant activity. Binding sites for radiolabelled dextromethorphan have been demonstrated in rat, guinea-pig and mouse brain and these sites are distinct from opiate receptors and, in particular, are not recognised by the competitive opiate antagonist, naloxone. However, the physiological relevance of these DM binding sites has still to be fully established although it is tempting to speculate that they are associated with the anticonvulsant and neuroprotective activities of DM. It seems plausible that activation of these binding sites mediates these pharmacological effects possibly via modulation of a voltage-operated calcium channel although a weak link with excitatory amino acid N-methyl-D-aspartate (NMDA) -gated channels has been proposed.

The purpose of the present study, which is continuing, is to examine fully the distribution of DM binding sites in normal rat brain and following ischaemic damage produced by middle cerebral artery occlusion. In addition, a full analysis of the distribution of these sites in post mortem human whole brain sections is underway although development of the technique in human brain tissue is still at an early stage.

Methods

[^{3}H]-Dextromethorphan receptor autoradiography - rat brain

Rats (Sprague-Dawley) were sacrificed by cervical dislocation and decapitation and the brains rapidly removed and frozen by immersion in isopentane cooled in liquid nitrogen. Frozen brains were stored at -80°C and subsequently equilibrated at -20°C for preparing cryostat sections (10-20µm). Each section was thaw-mounted on a glass slide previously coated with a solution of gelatine (0.5%) and chromic potassium sulphate (0.05%) in water (5 minutes immersion followed by drying in a stream of warm air). Slide-mounted sections were stored at -20°C until required. Thawed sections were immersed in buffer solution (Tris-HCl 50mM pH 7.5 or phosphate buffer pH 7.5 or Krebs' solution) at room temperature for 30 minutes and then allowed to dry. 350-500µl of buffer solution (normally Tris-HCl unless specified) containing 25nM [^{3}H]-dextromethorphan (83 Ci/mmol) was pipetted on to each slide to cover the brain section. For determining non-specific binding 100µM unlabelled DM was also present. The sections were incubated at room temperature or 4°C for 30 minutes unless otherwise stated. After incubation the solution was

aspirated off and the slides washed three times for 5 minutes in ice-cold buffer before drying under a stream of cool air. Dry slides were juxtaposed to tritium-sensitive film (Hyperfilm) for approximately 30 days before developing the film in D-19 developer. Films were analysed by Quantimet image analysis to determine the amount of radiolabelled DM bound to each section. Calibration standards on each film enabled a quantitative analysis to be made. As a preliminary to determine the conditions most suitable for obtaining optimal binding, sections were immersed in scintillation fluid and radioactivity levels determined in a scintillation spectrometer. Subsequently this also provided an indication of the length of time required for exposure of each film.

[^{3}H]-Dextromethorphan receptor autoradiography - human brain

Normal whole brains were obtained with a full history of each individual. Brains were sliced coronally at 1.5cm intervals whilst frozen at -20°C. Experiments performed so far, have employed 1-2cm square 30-40μm sections of cerebral cortex to provide the optimal conditions for binding. These experiments are still in progress. At the same time we have been developing a method for preparing whole brain sections to perform the autoradiographical analysis of [^{3}H]-DM binding. Coronal slabs of brain (1.5cm thick) are supported in a viscous solution of carboxy-methyl cellulose and frozen at -20°C. This frozen slab is then mounted on the stage of a PMV microtome for preparing 30-40μm sections. Fig.1a illustrates this arrangement where the brain block can be seen below and to the right of the knife-holder. Sections are cut at -20°C and mounted on a Hydrobond support membrane in preparation for incubation with [^{3}H]-DM. A section just obtained from the cryostat is shown in Fig.1b This section of frontal cortex is being held by hand to provide an indication of the size of the tissue. This is then dried and incubated by immersion in a solution containing [^{3}H]-DM. Preliminary data indicate that specific binding can be obtained in human brain but the ideal conditions have yet to be established. Using the incubation conditions established for rat brain sections has provided the starting point. However, the nature of the incubation buffer to be used has yet to be fixed as preliminary data indicate that the degree of specific binding differs depending on the buffer solution employed (see Fig.2). The maximal specific binding obtaining thus far is 23% of total which must be increased before attempting autoradiographic imaging. Further results are, therefore, not included in this report.

Autoradiography in brain sections from rats subjected to middle cerebral artery occlusion

Surgical Procedure

Male Sprague-Dawley rats (200-350g) were maintained on a 12 hour light/dark cycle and allowed access to food and water *ad libitum* both before and after the surgical procedure.

Rats were anaesthetised intraperitoneally with 60mgkg^{-1} sodium pentobarbitone and placed in a supine position on a heated operating table. The left and right common carotid arteries were exposed through a 1mm incision made in the midline of the neck and the vagal nerve carefully teased away from the blood vessels. A ligature was then placed loosely around each vessel.

The rat was then turned on its left side, and a 2cm vertical incision made midway between the right eye and right ear, taking care to avoid transection of the facial nerve. The temporalis muscle was cut with entomological scissors and watchmakers forceps in a manner to protect against traumatisation and bleeding of the tissue. The zygomatic arch and squamosal bone of the skull were then exposed under low power of the stereomicroscope (Zeiss Instruments). Using a saline-cooled dental drill, the exposed skull was removed to a thin layer above the middle cerebral artery. This layer was carefully peeled away using watchmakers forceps, leaving the middle cerebral artery clearly visible through the dura and arachnoid mater. At this point, the common carotid arteries were occluded; the right artery was permanently ligated, and the left artery occluded, using a small artery clip, for 60 minutes.

Using the tip of a fine needle and watchmakers forceps, the dura was carefully lifted away from the brain and cut to expose the middle cerebral artery. The surface of the brain was kept moistened with saline at all times. The arachnoid was parted and the middle cerebral artery was lifted slightly from the brain using the fine needle, and coagulated by passing an electric current (50mA) through the tips of microforceps placed along either side along a predetermined length of the vessel, for approximately 1-2mm below the level of the inferior cerebral vein. The artery was then cut to ensure complete occlusion. All visible branches of the middle cerebral artery were also occluded. The temporalis muscle was sutured separately from the overlying skin layers, and the wound cleaned up and treated with "Cicatrin" powder (antibacterial) and Xylocaine spray (local anaesthetic). In sham-operated animals, exactly the same procedure was followed, except that the dura was opened over the artery, but the artery was not occluded.

The rats were placed in an incubator at 32°C to maintain body temperature until recovery from anaesthesia was complete (usually within 2 hours). Temperature was monitored throughout the experiment, and maintained via adjustment of the heated table between 37.5°C and 38.5°C. Once recovery from anaesthesia was complete, the animals were returned to their cages, and maintained as described above. Body weight was monitored every 24 hours from the day of the experiment to the day of sacrifice.

The rats were sacrificed at 72 hours when the extent of neural damage in other rats had been fully determined by triphenyltetrazolium chloride staining. The brains were removed and frozen at -80°C for at least 24 hours. Sections (20μm) were cut at -20°C and the distribution of [^{3}H]-DM binding sites in the ipsilateral and contralateral sides of the brain determined as described above. Comparison with sham-operated as well as normal control rats was made.

Results - Rat brain

Effect of incubation time on ^{3}H-DM binding

The influence of the incubation time at room temperature (RT) and 4°C on ^{3}H-DM binding is illustrated in Fig.3 and Fig.4 In these experiments the wash time was fixed at 15 minutes (3 x 5 minutes). Comparable results were obtained at the two temperatures although the rate of association was, not surprisingly, faster at room temperature that at 4°C. However, equilibration was achieved in both cases by 30 minutes with at least 50% specific binding being obtaining throughout the incubation period.

Influence of washing time

The dissociation of ^{3}H-DM from sections of rat brain, by washing the sections for periods up to 60 minutes, is illustrated in Fig.5 This indicates the femtomoles of ^{3}H-DM specifically bound to whole parasaggital sections of rat brain (approx. 0.5mm from midline) after washing for 2.5, 5, 10, 15, 30 and 60 minutes in Tris HCl buffer at 4°C. The percentage specific binding at each time point is shown in the inset. Although the amount bound rapidly decreased the proportion specifically bound increased after 5 minutes. Thus, a 15 minute washing time was chosen for all subsequent studies.

Autoradiographical distribution of [^{3}H]-DM binding sites in rat brain

The distribution of [^{3}]-DM in normal rat brain is shown in Table 1. In general, binding was highest in the midbrain, hindbrain and the molecular cerebellar layer. High levels of [^{3}H]-DM binding were found in the dorsal raphe, rhabdoid nucleus and Purkinje cell layer of the cerebellum, while low levels were found in the olfactory bulb, thalamus, hypothalamus and the cerebellar molecular layer. Binding was high in the anterior amygdaloid nuclei, but was considerably lower in the medial basolateral amygdaloid nucleus. Intermediate levels of binding were found throughout the cortex of the brain, the striatum, hippocampus (CA1, CA2, CA3 regions, dentate gyrus) lateral geniculate nucleus and various structures of the midbrain (substantia nigra and tegmental nucleus), pons (pontine nucleus, locus coeruleus), and the granule cell layer

of the cerebellum. In the brain stem, binding was concentrated in the spinal trigeminal nucleus, but was very low in the spinal trigeminal tract.

Distribution of [^{3}H]-DM sites in rat brain following middle cerebral artery occlusion

Fig.6 illustrates the extent of the infarct in rat brain 72 hours after middle cerebral artery occlusion. The mean (± s.e.m.) area of the infarct was 61.0 ± 4.6 mm^2 in 6 rats which contrasted with 40.1 ± 2.9 after 24 hours (n=6). At 48 hours and 96 hours the areas were not significantly different (54.0 ± 6.9; n=7 and 55.6 ± 4.4; n=5, respectively) from the 72 hour value.

Tables 2 & 3 show the comparative densities of [^{3}H]-DM binding sites in sham-operated and middle cerebral artery occluded rat brain sections following incubation in 25nM [^{3}H]-DM. A reduced density of binding on the ipsilateral side of the occluded brains was noted particularly in the parietal cortex although a possible reduction was also apparent in other cortical regions. There were no obvious changes in the cingulate, frontal or entorhinal cortices and many other brain regions including the nucleus accumbens, globus pallidus, amygdala and lateral geniculate. Overall little or no changes in binding occurred in regions outside the infarct region. Presumably the reductions which did occur reflect the necrosis of cells in those regions. However, the loss of binding was not as great as might be expected in those regions if the binding is simply a marker of neuronal integrity. It seems possible that DM may bind to an intracellular component of neurones i.e. microsomal rather than the synaptosomal fraction to which most neurotransmitter binding occurs., Perhaps cellular necrosis does not alter this, at least within the 72 hour period studied so far.

As mentioned in the Introduction, it has been suggested that there may be a functional relationship between DM sites and the NMDA receptor. However, the distribution of [^{3}H]-DM binding sites observed in the present study do not correlate with the known distribution of NMDA binding sites. This does not rule out the existence of a functional interaction between DM and NMDA in discrete regions of the brain but it does support the view that DM can exert its effects independently of the NMDA receptor complex.

In summary, we have defined the distribution of binding sites for DM in the normal rat brain as well as in brains obtained from animals 2 hours after middle cerebral artery occlusion. The occlusion had only limited influence on the overall distribution of binding sites. A technique for examining the distribution of [^{3}H]-DM binding sites in post mortem human whole brain sections has been developed and future studies will be devoted to establishing the pattern of binding in both normal and diseased brains.

TABLE 1 Autoradiographical distribution of [^{3}H]-DM binding sites in rat brain

Area	[^{3}H]-DM Bound (fmol/mg tissue)	n
Olfactory bulb:		
Olfactory bulb	18.8 ± 3.0	4
AOD	19.8 ± 2.8	4
Cerebral Cortex:		
cingulate	38.6 ± 9.7	4
frontal	36.3 ± 6.8	4
parietal	37.6 ± 8.5	4
insular	40.0 ± 5.1	4
piriform	39.8 ± 4.8	4
retrosplenial	41.4 ± 8.8	4
occipital	38.2 ± 6.8	4
temporal	37.2 ± 7.8	4
perirhinal	35.6 ± 10.1	4
entorhinal	30.1 ± 6.7	4
Basal Ganglia:		
caudate putamen	35.1 ± 6.8	4
nucleus acumbens	40.3 ± 7.3	4
globus pallidus	41.9 ± 11.6	4
Septum:		
lateral septal nucleus	33.2 ± 5.2	4
Amygdala:		
anterior amygdaloid nucleus	45.6 ± 10.8	4
basolateral amygdaloid nucleus	20.3 ± 3.7	4
Hippocampus:		
CA1	39.5 ± 9.7	4
CA2	42.9 ± 8.2	4
CA3	44.2 ± 8.6	4
dentate gyrus	43.2 ± 11.9	4
Thalamus:		
ventromedial thalamus	29.8 ± 5.6	4
laterodorsal thalamus	28.4 ± 5.2	4
lateral geniculate nucleus	47.4 ± 8.8	4
Hypothalamus:		
ventromedial hypothalamus	21.8 ± 3.7	4

TABLE 1 (continued)

Area	$[^3H]$-DM Bound (fmol/mg tissue)	n
Midbrain:		
central grey	57.5 ± 10.5	4
superior colliculus	54.9 ± 15.6	4
inferior colliculus	32.8 ± 11.9	4
caudal linear raphe nucleus	49.4 ± 8.5	4
dorsal raphe nucleus	72.4 ± 28.9	4
median raphe nucleus	53.8 ± 27.2	4
rhaboid nucleus	62.7 ± 11.4	4
interpeduncular nucleus	50.1 ± 9.1	4
substantia nigra	48.5 ± 13.2	4
Cranial Nerve Nuclei:		
spinal trigeminal nuclei	55.3 ± 11.1	4
spinal trigeminal tract	20.3 ± 2.6	4
Pons:		
pontine nucleus	42.4 ± 3.4	4
locus coeruleus	35.1 ± 3.4	4
dorsal tegmental nuclei	42.7 ± 12.2	4
raphe points	37.4 ± 13.6	4
Medulla Oblongata:		
nucleus solitary tract	48.5 ± 9.5	4
brainstem reticular nucleus	53.7 ± 13.0	4
inferior olive	48.2 ± 4.4	4
Cerebellum:		
Purkinje cell layer	64.0 ± 10.5	4
granular cell layer	46.1 ± 7.5	4
molecular cell layer	23.4 ± 1.1	4

TABLE 2: Autoradiographical distribution of [^{3}H]-DM binding sites in the sham-operated rat brain.

Brain Area	[^{3}H]-DM Bound (fmol/mg)		n
	contralateral	ipsilateral	
Cerebral Cortex:			
cingulate	32.4 ± 7.1	32.9 ± 7.0	5
frontal	32.4 ± 7.9	32.6 ± 6.2	5
parietal	34.3 ± 6.2	36.2 ± 5.8	5
insular	25.5 ± 8.9	32.5 ± 6.6	5
retrosplenial	30.0 ± 8.9	26.5 ± 7.9	5
occipital	29.8 ± 9.6	31.7 ± 7.0	5
temporal	35.0 ± 10.5	35.9 ± 7.7	5
perirhinal	31.3 ± 9.6	28.6 ± 6.8	5
entorhinal	27.6 ± 9.0	22.2 ± 7.1	5
Basal Ganglia:			
caudate putamen	30.5 ± 7.2	31.4 ± 6.0	5
nucleus acumbens	31.4 ± 8.2	34.2 ± 8.1	5
globus pallidus	37.7 ± 7.7	36.5 ± 7.0	5
Septum:			
lateral septal nucleus	22.1 ± 4.5	21.0 ± 4.8	5
Amygdala:			
basolateral amygdalioid nucleus	33.8 ± 8.5	34.4 ± 6.9	5
Hippocampus:			
CA1	34.8 ± 7.1	37.4 ± 4.8	5
CA2	40.1 ± 7.6	40.9 ± 6.3	5
CA3	41.1 ± 7.5	41.8 ± 6.7	5
dentate gyrus	35.7 ± 7.7	34.1 ± 7.0	5
Thalamus:			
laterodorsal thalamus	44.1 ± 8.7	43.9 ± 8.2	5
mediodorsal thalamus	46.7 ± 8.6	46.7 ± 8.9	5
ventral thalamus	42.1 ± 8.6	43.7 ± 8.2	5
geniculate nucleus	42.7 ± 8.9	41.5 ± 9.3	5
Hypothalamus:			
ventromedial hypothalamus	39.8 ± 5.6	40.8 ± 6.4	5

TABLE 2 (continued)

Brain Area	$[^3H]$-DM Bound (fmol/mg)	n
Midbrain:		
central grey	35.8 ± 7.9	5
caudal linear raphe nucleus	36.8 ± 10.1	5
dorsal raphe nucleus	47.8 ± 9.3	5
median raphe nucleus	33.7 ± 7.4	5
rhaboid nucleus	42.8 ± 7.1	5
Medulla Oblongata:		
nucleus solitary tract	36.8 ± 7.1	5
reticular nucleus	36.6 ± 7.6	5
Cerebellum:		
Purkinje cell layer	48.6 ± 10.3	5
granular cell layer	28.6 ± 7.8	5
molecular cell layer	15.4 ± 4.4	5

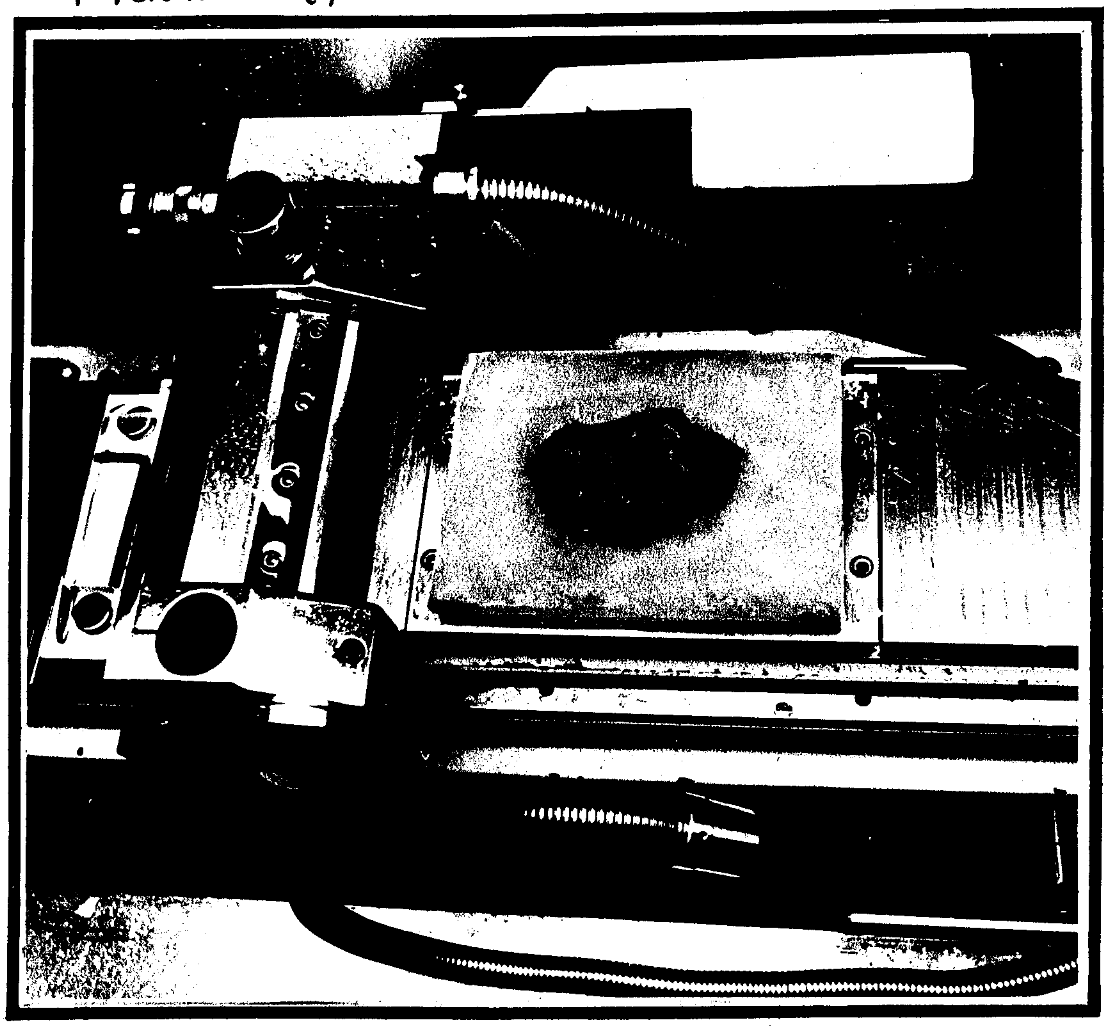
TABLE 3: Autoradiographical distribution of [^{3}H]-DM binding sites in the MCA-occluded rat brain

Brain Area	[^{3}H]-DM Bound (fmol/mg)		n
	contralateral	ipsilateral	
Cerebral Cortex:			
cingulate	44.9 ± 8.5	43.1 ± 8.7	5
frontal	42.7 ± 7.3	35.2 ± 8.5	5
parietal	43.1 ± 7.8	21.8 ± 6.4	5
insular	33.9 ± 5.8	29.7 ± 8.4	5
retrosplenial	32.5 ± 7.3	32.5 ± 7.4	5
occipital	26.1 ± 7.8	17.4 ± 3.6	5
temporal	28.8 ± 9.0	20.2 ± 4.1	5
perirhinal	22.0 ± 5.2	22.4 ± 3.3	5
entorhinal	22.4 ± 6.0	26.2 ± 3.3	5
Basal Ganglia:			
caudate putamen	43.9 ± 8.1	41.4 ± 8.7	5
nucleus acumbens	37.8 ± 35.1	35.1 ± 8.4	5
globus pallidus	34.9 ± 2.5	36.3 ± 2.9	5
Septum:			
lateral septal nucleus	34.0 ± 6.9	34.7 ± 6.3	5
Amygdala:			
basolateral amygdalioid nucleus	20.6 ± 7.2	22.5 ± 5.6	5
Hippocampus:			
CA1	23.3 ± 6.0	26.9 ± 6.5	5
CA2	28.8 ± 6.9	34.9 ± 7.8	5
CA3	30.3 ± 8.2	33.9 ± 8.0	5
dentate gyrus	30.8 ± 36.2	36.2 ± 3.7	5
Thalamus:			
laterodorsal thalamus	32.8 ± 10.7	35.0 ± 9.6	5
mediodorsal thalamus	31.8 ± 8.2	32.9 ± 8.8	5
ventral thalamus	32.9 ± 8.8	33.6 ± 10.0	5
geniculate nucleus	43.5 ± 9.7	51.3 ± 7.7	5
Hypothalamus:			
ventromedial hypothalamus	26.6 ± 8.1	30.9 ± 8.1	5

TABLE 3 (continued)

Brain Area	[^{3}H]-DM Bound (fmol/mg)	n
Midbrain:		
central grey	39.1 ± 4.0	5
caudal linear raphe nucleus	46.7 ± 4.3	5
dorsal raphe nucleus	47.5 ± 4.1	5
median raphe nucleus	41.5 ± 5.2	5
rhaboid nucleus	45.5 ± 4.1	5
Medulla Oblongata:		
nucleus solitary tract	32.7 ± 7.4	5
reticular nucleus	34.7 ± 6.8	5
Cerebellum:		
Purkinje cell layer	43.0 ± 7.4	5
granular cell layer	23.0 ± 7.3	5
molecular cell layer	14.0 ± 4.4	5

FIGURE 1(a)



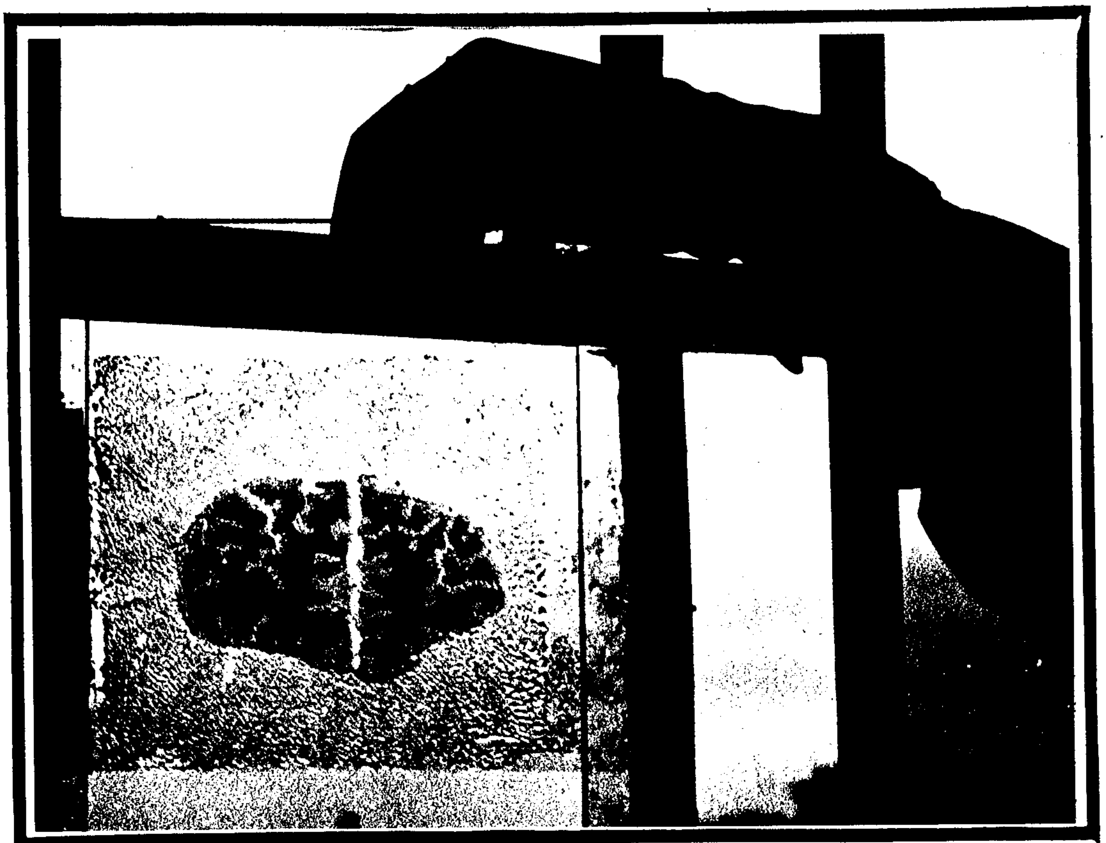


FIGURE 1(b)

FIGURE 2

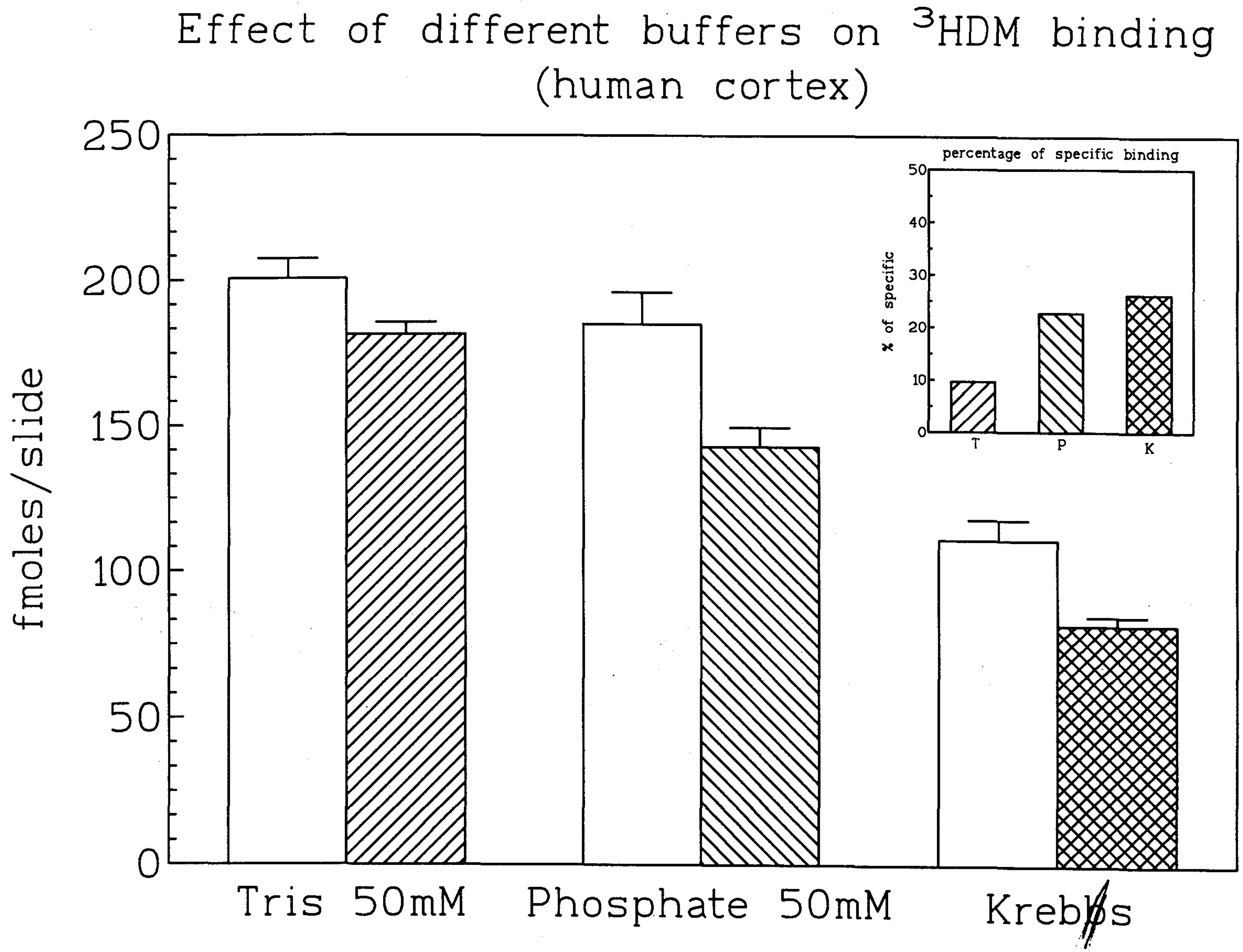
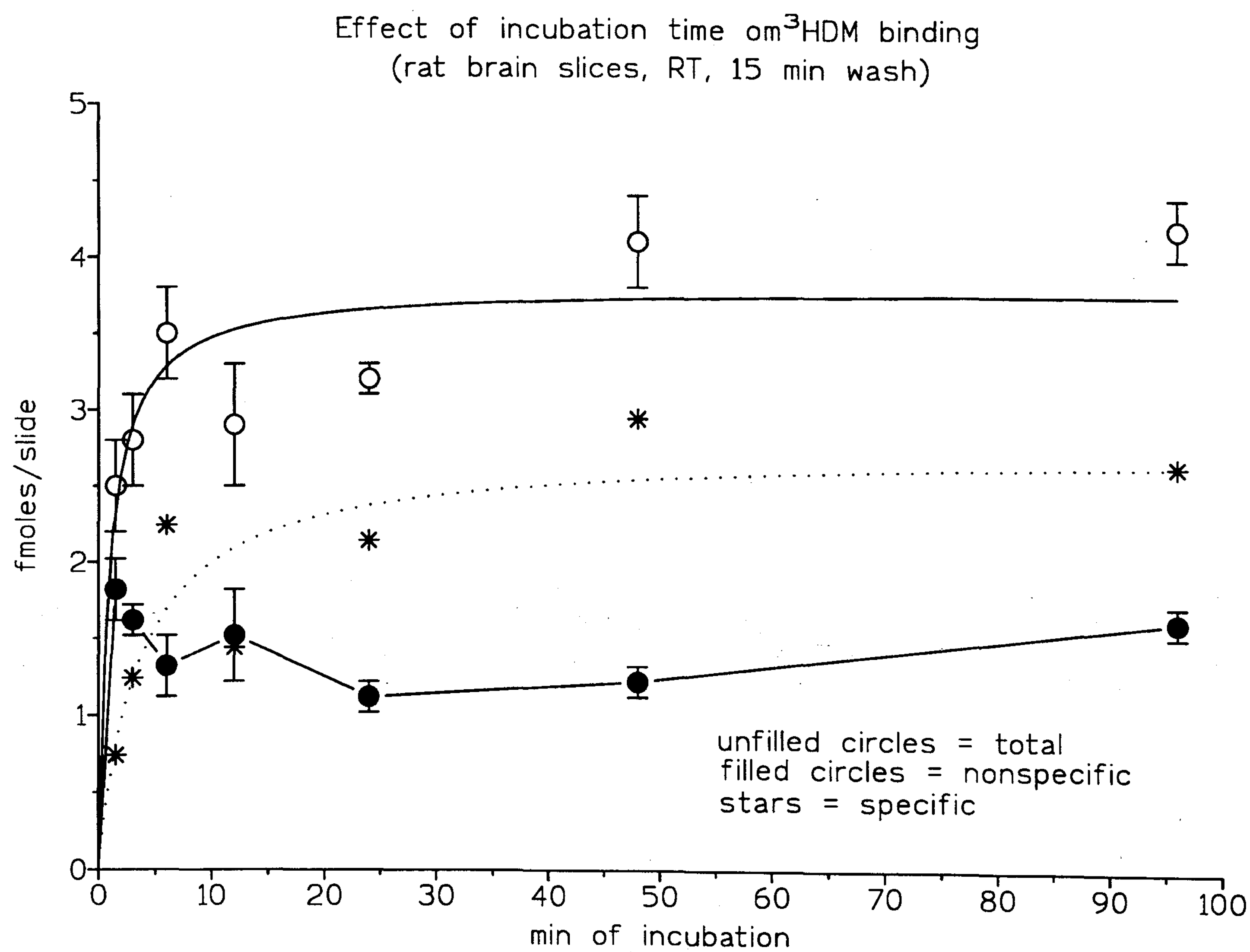

Effect of different buffers on ³HDM binding
(human cortex)
fmoles/slide
250
200
150
100
50
0
Tris 50mM
Phosphate 50mM
Krebs
percentage of specific binding
% of specific
50
40
30
20
10
0
T
P
K

FIGURE 3



Effect of incubation time om^{3}HDM binding
(rat brain slices, RT, 15 min wash)
fmoles/slide
0
1
2
3
4
5
0
10
20
30
40
50
60
70
80
90
100
min of incubation
unfilled circles = total
filled circles = nonspecific
stars = specific

FIGURE 4

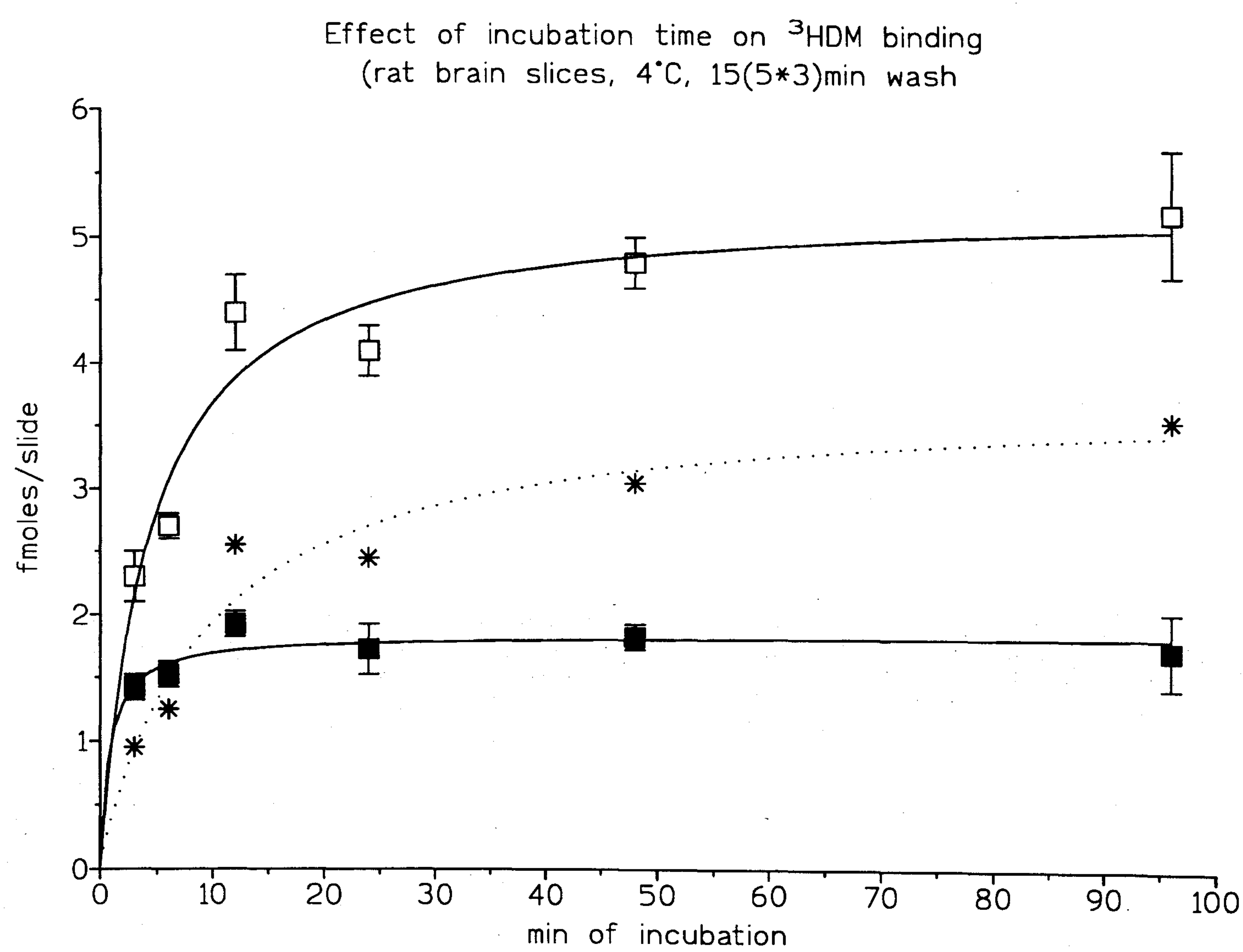
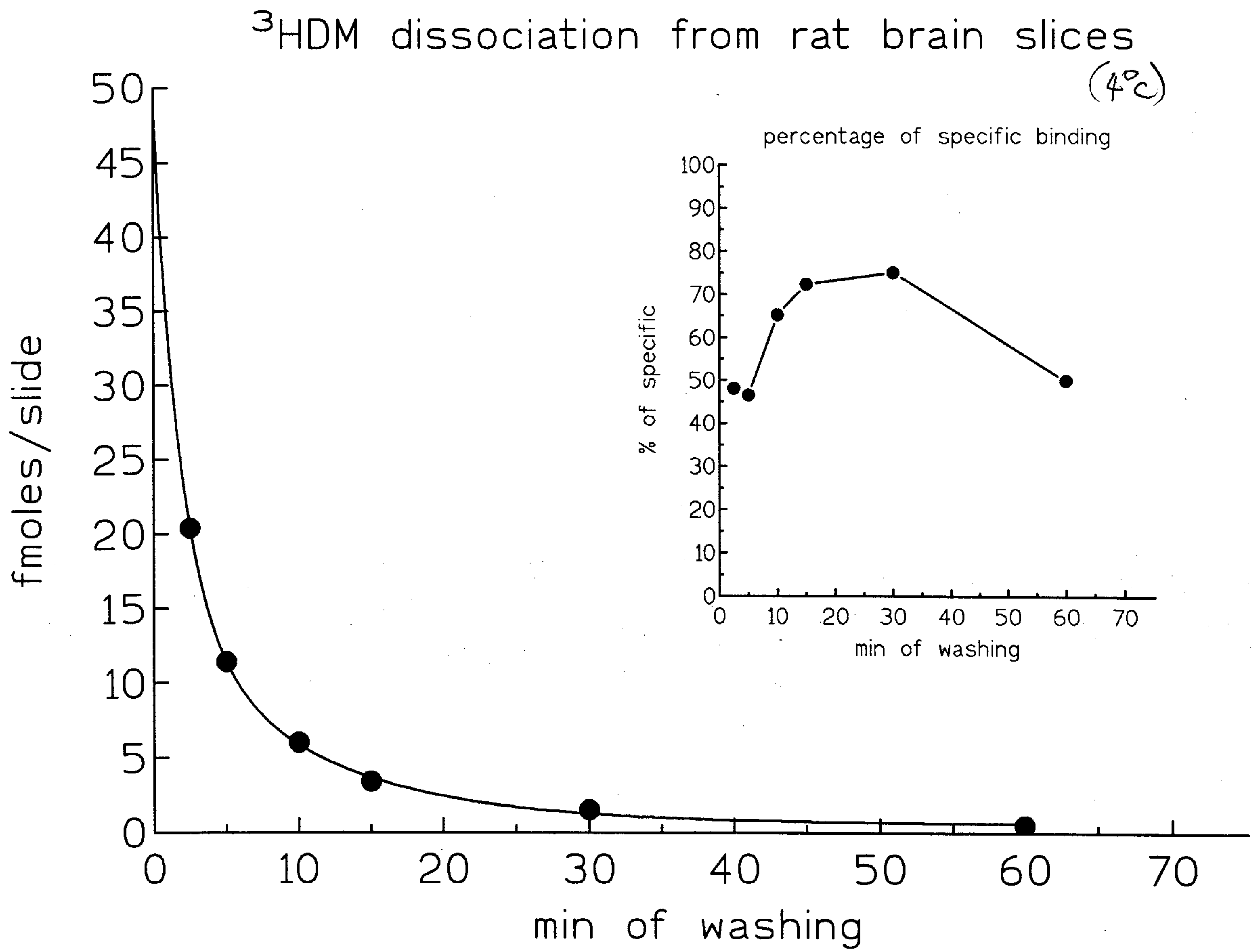

Effect of incubation time on ³HDM binding
(rat brain slices, 4°C, 15(5*3)min wash
fmoles/slide
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90
100
min of incubation

FIGURE 5



³HDM dissociation from rat brain slices
(4°C)
fmoles/slide
0
5
10
15
20
25
30
35
40
45
50
0
10
20
30
40
50
60
70
min of washing
percentage of specific binding
% of specific
0
10
20
30
40
50
60
70
80
90
100
min of washing